JOURNAL OF BACTERIOLOGY, Apr. 1990, p. 1962–1968
0021-9193/90/041962-07$02.00/0

Vol. 172, No. 4

Efficiency Factors and ATP/ADP Ratios in Nitrogen-Fixing *Bacillus polymyxa* and *Bacillus azotofixans*†

KEIKO KANAMORI,[1,2]‡* RICHARD L. WEISS,[1] AND JOHN D. ROBERTS[2]

Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024-1569,[1] and Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91125[2]

Received 6 September 1989/Accepted 16 January 1990

The efficiency factor, the number of moles of ATP generated per mole of glucose fermented, was determined in anaerobic, non-carbon-limited N_2-fixing cultures of *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus azotofixans*, and *Clostridium butyricum* through identification and quantitation of the fermentation products by ^{13}C nuclear magnetic resonance spectroscopy and measurement of acetate kinase activities. All three *Bacillus* species had acetate kinase activities and produced acetate and ethanol as the major fermentation products. The maximum amounts of ATP generated per mole of glucose fermented were 2.70, 2.64, and 2.88 mol in *B. polymyxa*, *B. macerans*, and *B. azotofixans*, respectively, compared with 3.25 mol in *C. butyricum*. Thus, in the N_2-fixing *Bacillus* species, the efficiency factors are lower than that in *C. butyricum*. Steady-state ATP/ADP concentration ratios were measured in non-carbon-limited N_2-fixing cultures of *B. polymyxa* and *B. azotofixans* through separation and quantitation of the adenylates in cell extracts by ion-pair reversed-phase high-performance liquid chromatography. The observed ATP/ADP ratios were 4.5 and 3.8, and estimated energy charges were 0.81 to 0.86 and 0.81 to 0.83, respectively, for *B. polymyxa* and *B. azotofixans*. The results suggest that under these growth conditions, the rate of ATP regeneration is adequate to meet the energy requirement for N_2 fixation in the *Bacillus* species, in contrast to N_2-fixing *Clostridium pasteurianum* and *Klebsiella pneumoniae*, for which substantially lower steady-state ATP/ADP ratios and energy charges have been reported. Implications of the results are discussed in relation to possible differences between *Bacillus* and *Clostridium* species in energy requirements for N_2 fixation and concomitant ammonia assimilation.

In a large variety of non-nitrogen-fixing cells grown under nonlimiting substrate conditions, Atkinson found that metabolically available energy defined as energy charge EC = ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]) falls within the range of 0.8 and 0.95 (2, 4). Typical ATP/ADP ratios in these cells were 4 to 6. The relative constancy of EC is attributed to regulation by adenylates of the activities of some enzymes in the ATP-regenerating and ATP-utilizing sequences. When the supply of exogenous substrate is adequate, any tendency for EC to fall is counteracted by an increase in the rate of ATP-regenerating sequences, thus tending to stabilize EC (2).

However, in nitrogen-fixing bacteria, *Clostridium pasteurianum* and *Klebsiella pneumoniae*, grown with nonlimiting substrates, Upchurch and Mortenson (26) observed low EC values (0.6 and 0.7, respectively) and low ATP/ADP ratios (1.4 and 2.0, respectively). In these cultures, the rate of ATP regeneration was considered to be growth rate limiting because of the high ATP requirement for N_2 fixation (estimated to be 20 and 29 mol of ATP per mol of N_2 fixed, respectively, for *C. pasteurianum* [6] and *K. pneumoniae* [8]). At the observed intracellular ATP/ADP ratios, nitrogenase, whose activity is strongly inhibited at low ATP/ADP ratios in vitro, was estimated to be operating at only 32% of its potential maximum in the cell. For this reason, it was suggested that the rate of ATP expenditure for N_2 fixation is regulated through partial inhibition of the nitrogenase activity and that low EC and ATP/ADP ratios are normal manifestations of an N_2-fixing physiology (26).

N_2-fixing *Bacillus* species, *B. polymyxa*, *B. macerans*, and *B. azotofixans*, provide interesting models for the study of cellular energy states during N_2 fixation because they are known, or expected, to differ from *C. pasteurianum* in some of the ATP-regenerating and ATP-utilizing metabolic pathways and hence in the rates of energy production and utilization. The rate of ATP regeneration in an anaerobic N_2-fixing culture grown on glucose as the carbon source depends on the rate of glucose fermentation and the efficiency factor (the number of moles of ATP generated per mole of glucose fermented). The efficiency factor in the *Bacillus* species is expected to be lower than that for *C. pasteurianum*, but it has not been determined. The fermentation of 1 mol of glucose to 2 mol of acetyl coenzyme A (acetyl-CoA) yields 2 mol of ATP, but it is not known whether additional ATP molecules are produced in the N_2-fixing *Bacillus* cultures through the following reactions:

$$\text{acetyl-CoA} + P_i \underset{}{\overset{\text{phosphotransacetylase}}{\rightleftharpoons}} \text{acetyl phosphate} + \text{CoA}$$

$$\text{acetyl phosphate} + \text{ADP} \overset{\text{acetate kinase}}{\rightleftharpoons} \text{acetate} + \text{ATP}$$

B. polymyxa ferments glucose to ethanol and (*R*,*R*)2,3-butanediol in acidic media (29) and to ethanol and acetate in glucose-limited culture grown with ammonia as the nitrogen source (1). The efficiency factor can thus range from 2, when no acetate is produced or acetate kinase is absent or both, to 3, which is the maximum value compatible with maintenance of redox balance and is attained when all of the glucose is fermented to equimolar quantities of acetate and ethanol.

* Corresponding author.

† Contribution no. 8023 from the Gates and Crellin Laboratories of Chemistry.

‡ Present address: Magnetic Resonance Spectroscopy Laboratory, Huntington Medical Research Institutes, 660 South Fair Oaks Ave., Pasadena, CA 91105.

For the recently characterized *B. azotofixans* (21), neither the fermentation products nor the efficiency factor is known.

The rates of ATP utilization for N_2 fixation and concomitant ammonia assimilation depend on the concentration and in vivo activity of nitrogenase, the number of moles of ATP consumed per mole of N_2 fixed, and the ATP requirement for ammonia assimilation. For *B. polymyxa* and *B. macerans*, which fix N_2 slowly and assimilate ammonia into glutamic acid mainly through the glutamate dehydrogenase pathway without expenditure of energy (12, 13), the maximum rate of ATP utilization for nitrogen assimilation may be lower than that for *B. azotofixans*, a moderate N_2 fixer, or *C. pasteurianum* and *K. pneumoniae*, efficient N_2 fixers, which expend additional ATP in assimilating ammonia through the glutamine synthetase-glutamate synthase pathways (3, 14, 18, 19). These considerations prompted us to investigate (i) the efficiency of ATP formation in N_2-fixing cultures of *B. polymyxa*, *B. macerans*, and *B. azotofixans* and (ii) the steady-state ATP/ADP ratios in N_2-fixing cultures of *B. polymyxa* and *B. azotofixans* for comparison with those reported for *C. pasteurianum* and *K. pneumoniae* (26).

MATERIALS AND METHODS

Strains, media, and growth. For determination of efficiency factors, N_2-fixing cultures of *B. polymyxa* ATCC 8519, *B. macerans* ATCC 8515, and *B. azotofixans* ATCC 35681 were grown anaerobically with 60 mM sucrose as the carbon source (instead of glucose to minimize slime formation), and *C. butyricum* ATCC 8260 was grown with 120 mM glucose, as described previously (12–15). Growth was monitored in a Klett-Summerson colorimeter with a no. 540 filter. At mid-exponential phase, the cells were harvested by centrifugation. The growth medium containing accumulated fermentation products was stored at −20°C until analysis. The cell pellet was used for acetate kinase assay.

For analysis of adenylates, N_2-fixing cultures of *B. polymyxa* and *B. azotofixans* were grown as described above, except that the sucrose concentration was 90 mM. For anaerobic ammonia-grown cultures, the medium was supplemented with 22 mM NH_4Cl. At mid-exponential phase, the cultures were sampled anaerobically for extraction of adenylates, as described below. The dry weight of cells was measured in duplicate for 20 ml of culture by the method of Herbert et al. (7).

Acetate kinase assay. For acetate kinase assay, the cell pellet was washed with 50 mM K_2HPO_4 buffer (pH 7.5) containing 0.004% sodium thioglycolate, suspended in 50 mM K_2HPO_4 buffer (pH 7.5) containing 5 mM mercaptoethanol, and disrupted by sonication. After the removal of cell debris by centrifugation, the supernatant containing 5 to 20 mg of protein per ml was used for the assay.

Acetate kinase activity was measured in nanomoles of acetyl phosphate formed per minute per milligram of protein by converting acetyl phosphate to its ferric hydroxamate complex and measuring the A_{540} as described in method c of Nakajima et al. (20), with the following modification. The reaction was terminated at various times after the addition of the cell extract, and the activity was calculated from the initial rate of formation of the complex at 37°C. Protein was measured by the method of Lowry et al. (17), with bovine serum albumin as the standard.

^{13}C NMR analysis of the fermentation products. Fermentation products in the growth medium were identified and quantitated by natural-abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The NMR sample was prepared by adding 0.2 ml of ^2H_2O for field-frequency lock to 2 ml of the growth medium. EDTA (8 mg) was also added to complex trace paramagnetic metal ions which might otherwise affect the line widths of the carbon resonances of the fermentation products. The ^{13}C NMR spectra were obtained on a Bruker AM-500 spectrometer operating at 125.79 MHz. The operating conditions employed 16-μs (90° flip angle) pulse width, 4-s delay, and proton decoupling by WALTZ-16 composite pulse sequence. ^{13}C chemical shifts are reported in parts per million downfield from tetramethylsilane. The fermentation products were identified by comparing their chemical shifts with those of the standards, viz. ethanol, sodium acetate, acetone, (*R*,*R*)2,3-butanediol, or sodium succinate, dissolved in sterile culture medium. Each fermentation product was quantitated by comparing the peak intensity (in integrated area) of its methyl or methylene carbon with the corresponding peak intensity observed for 800 μmol of the standard in sterile medium, prepared for NMR as described above and measured under identical NMR operating conditions. The peak intensity of a carbon depends not only on its quantity but also on its spin lattice relaxation time (T_1) and nuclear Overhauser effect. However, quantitation by this method was expected to be accurate, because the effects of T_1 on, and the contribution of the nuclear Overhauser effect to, peak intensity are the same for the standard and the corresponding fermentation product. To evaluate the accuracy of this method, butyrate in the growth medium of *C. butyricum* was quantitated separately from the observed peak intensities of C-2, C-3, and C-4. The quantities of butyrate calculated from the three carbon peaks agreed to within 4%, demonstrating the accuracy of the method.

Calculation of efficiency factor. The efficiency factor, as defined by Tempest and colleagues (5, 24), is the number of moles of ATP generated per mole of glucose fermented. It is to be distinguished from the number of moles of ATP generated per mole of glucose consumed (which is the sum of the quantity of glucose fermented and the quantity of glucose assimilated into biomass), a quantity often measured (22), because it is used for the calculation of Y_{ATP} (grams [dry weight] of cells per mole of ATP) from Y_{glc} (grams [dry weight] of cells per mole of glucose consumed), but not referred to as an efficiency factor. The efficiency factor was calculated on the assumption, on which all reported values of efficiency factor and Y_{ATP} are based (5, 24), that the coupling between ATP regeneration and the formation of fermentation products is complete. Therefore, for organisms in which glucose is catabolized by the Embden-Meyerhof pathway, it is assumed that 2 mol of ATP is regenerated for each mole of glucose that is fermented to 2 mol of acetyl-CoA and that an additional mole of ATP is regenerated per mole of acetate produced, if acetate kinase activity is present.

The efficiency factor was calculated from the observed quantities of fermentation products on the basis of the known stoichiometries of the fermentation pathways as follows. Let P = moles of glucose carbon fermented ($P/6$ = moles of glucose fermented). Q_A = moles of acetate carbon produced ($Q_A/2$ = moles of acetate produced). Q_B, Q_C, . . . Q_N = moles of carbon in all other non-gaseous fermentation products, B, C, . . . N, respectively.

If the formation of B, C, . . ., N from acetyl-CoA does not involve net ATP formation, which was found to be true for the *Bacillus* species studied here (see Results), then the efficiency factor, the number of moles of ATP produced per mole of glucose fermented, is given by

$$\text{Efficiency factor} = [2(P/6) + Q_A/2]/(P/6) = 2 + 3(Q_A/P) \quad (1)$$

In the *Bacillus* species, 1 mol of glucose is converted to 2 mol of pyruvate and then to 2 mol each of acetyl-CoA and formate by pyruvate-formate lyase, and 2 mol of formate is, in turn, converted to 2 mol each of CO_2 and H_2 by formate-hydrogen lyase complex (28). Thus, two of six glucose carbons are lost as CO_2. This means that the number of moles of glucose carbon fermented can be related to the number of moles of non-gaseous product carbons by

$$P = 3(Q_A + Q_B + \ldots + Q_N)/2 \quad (2)$$

Substituting equation 2 into equation 1, we obtain

$$\text{Efficiency factor} = 2 + 2[Q_A/(Q_A + Q_B + \ldots\ldots + Q_N)] \quad (3)$$

Therefore, the efficiency factor can be determined from the fraction of acetate carbon in the total non-gaseous product carbon, without direct measurement of the quantity of glucose fermented.

In studies of anaerobic chemostat cultures of *Klebsiella aerogenes* by Teixeira de Mattos and Tempest (24), the quantity of glucose fermented was taken to be the difference between the experimentally measured quantity of total glucose consumed and the quantity of glucose assimilated into biomass (as estimated from the carbon content of the dry weight of the biomass). This method is convenient when determination of Y_{ATP} and Y_{glc} as well as the efficiency factor is desired. However, for the calculation of the efficiency factor alone, a careful comparison of their methods and ours showed that both are based on the assumptions described above and yield the same values of the efficiency factor for given quantities of fermentation products.

High-performance liquid chromatography analysis of adenylates. The adenylates were extracted by $HClO_4$ as described by Walker-Simmons and Atkinson (27), with the following modification to ensure anaerobic sampling. A 1-ml sample of the culture was transferred rapidly (within 2 s) and anaerobically, either through the sampling port (N_2-fixing culture) or with a syringe inserted through the rubber septum covering the culture flask (anaerobic ammonia-grown cultures), to a 0.2-ml sample of cold 35% (wt/vol) $HClO_4$. The adenylates were extracted by stirring the mixture in an ice bath for 30 min. After centrifugation at 17,200 × *g* to precipitate cell debris, the supernatant was neutralized to pH 6.9 with 0.25 ml of an aqueous solution containing 2.6 M KOH and 0.58 M $KHCO_3$. After the precipitate of $KClO_4$ was removed by centrifugation, the supernatant containing adenylates was filtered through a 0.2-μm (pore-size) membrane in a disposable filter assembly (Gelman Sciences, Inc., Ann Arbor, Mich.) and stored at −80°C until analysis. A high-performance liquid chromatography sample contained a measured volume (100 to 200 μl) of the cell extract to which 220 mM potassium phosphate buffer (pH 6.9) was added to a final volume of 220 μl, and tetrabutylammonium hydrogen sulfate was added to a final concentration of 0.7 mM. The mixture was maintained at room temperature for 30 min to permit complexation of the pairing ion with adenylates before injection.

The adenylates were separated and quantitated by ion-pair reversed-phase high-performance liquid chromatography, which permits selective retention of adenine nucleotides (10). The chromatograph consisted of a pump (model 6000A; Waters Associates, Inc., Milford, Mass.) and an injector (model 7010; Rheodyne, Berkeley, Calif.). The separation was carried out on a 5-μm Supelcosil LC-18 (25 by 0.46 cm) analytical column protected with a 5-μm Supelguard LC-18 guard column (2 by 0.46 cm) (Supelco, Bellefonte, Pa.). The chromatographic solvent was an aqueous solution of 220 mM potassium phosphate (pH 6.9) containing 0.3 mM tetrabutylammonium hydrogen sulfate and 1% (vol/vol) methanol. The flow rate was 1.3 ml/min at 1,600 lb/in^2. The eluate was monitored at 254 nm with a UV detector (model 440; Waters Associates). The detector response was recorded on either a recorder (model SR-204; Heath Co., Benton Harbor, Mich.) or a plotter (Chromatopac CR3A; Shimadzu, Kyoto, Japan) equipped with an electronic integrator for computation of peak areas. A linear correlation between the peak area and the quantity injected was observed for 0.05 to 1.0 nmol of AMP, ADP, and ATP dissolved in the chromatographic solvent. This range includes the amount actually injected when cell extracts were analyzed.

The quantities (nanomoles) of ADP and ATP in the injected cell extract were determined from the peak areas by calibration with the standards. The concentrations of ATP and ADP (in nanomoles per milliliter of culture) were calculated after taking into account the injected volume of the cell extract and the dilution factor (to 1 ml of the culture was added 0.2 ml of $HClO_4$ for extraction and 0.25 ml of the KOH-$KHCO_3$ solution for neutralization).

After 15 to 20 injections, the column was cleaned by a 90-min water wash, followed by a 30-min methanol (100%) wash and a 90-min water wash, and reequilibrated with the chromatographic solvent for several hours. With such treatment, the retention times of the adenylate standards remained highly reproducible.

RESULTS

Efficiency factor. Figure 1 shows the ^{13}C NMR spectra of the growth medium of N_2-fixing cultures of *B. polymyxa*, *B. macerans*, *B. azotofixans*, and *C. butyricum* containing the fermentation products as well as the residual nutrients. With *B. polymyxa*, ethanol and acetate are the major fermentation products and (*R*,*R*)2,3-butanediol and succinate are minor products. ATP is not produced during the formation of ethanol from acetyl-CoA (25) nor during the formation of (*R*,*R*)2,3-butanediol from two pyruvate molecules with the release of two CO_2 molecules (9). Fumarate is known to be reduced to succinate by NADH, but this reaction does not involve net production of ATP (23). Consequently, acetate is the only product whose formation from acetyl-CoA can result in ATP production.

With *B. macerans*, ethanol and acetate are the major products in exponentially growing N_2-fixing cultures; acetone, a minor product in ammonia-grown cultures (28), was not produced. With *B. azotofixans*, ethanol and acetate are the major products and lactate is a minor product. The concentrations of accumulated fermentation products, determined from the spectra as described in Materials and Methods, are listed in Table 1.

Acetyl-CoA is converted to acetate with production of ATP by the action of acetate kinase. Assays of cell extracts of N_2-fixing *Bacillus* species showed that all three species have acetate kinase activity (Table 1).

The efficiency factor was calculated from the observed fraction of acetate carbon in the total non-gaseous product carbon as described in detail in Materials and Methods. With the *Bacillus* species, sucrose (the carbon source used to minimize slime formation) is hydrolyzed, after uptake, to glucose and fructose. ATP (2 mol) is regenerated during fermentation of 1 mol of glucose (or fructose) to 2 mol of

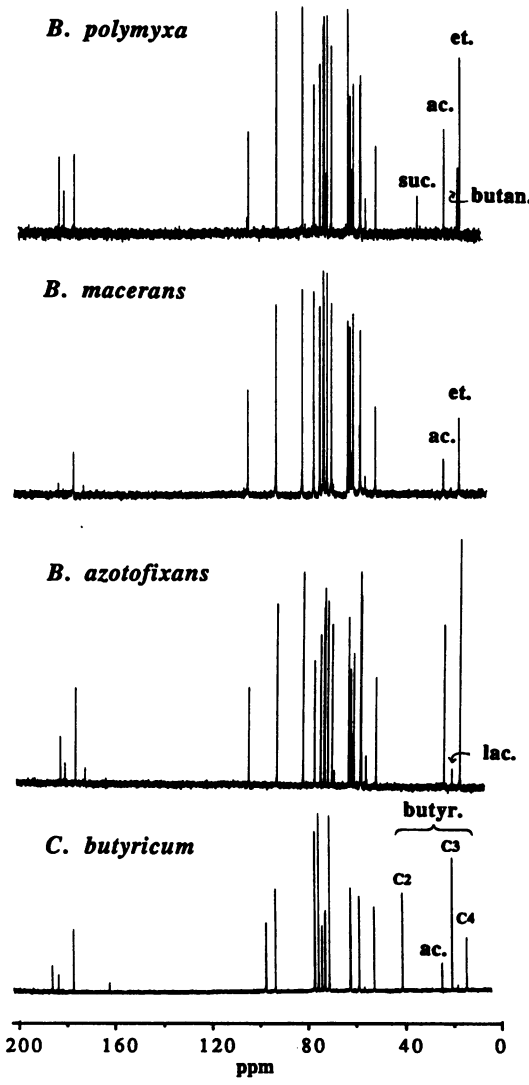

FIG. 1. ^{13}C NMR spectra of the growth media of N_2-fixing cultures of *B. polymyxa*, *B. macerans*, *B. azotofixans*, and *C. butyricum* containing the fermentation products. The methyl or methylene carbon peak of each product used for quantitation is labeled with the following abbreviations. ac., Acetate C-2; butan., (*R*,*R*)2,3-butanediol C-2 and C-3; butyr., butyrate C-2, C-3, and C-4; et., ethanol C-2; lac., lactate C-3; suc., succinate C-2 and C-3. Peaks in the 50 to 105-ppm region represent carbons of residual sucrose (*Bacillus* spp.) or glucose (*C. butyricum*). Peaks at 176.4, 182.6, and 185.0 ppm represent the carboxyl carbons of EDTA, acetate, and butyrate, respectively.

acetyl-CoA. With *B. macerans*, the fraction of acetate carbon in the total non-gaseous product carbon is 0.32 (Table 1). Therefore, the efficiency factor, by equation 3, is 2 + [2 × (0.32)] = 2.64 mol of ATP per mol of glucose fermented. With *B. polymyxa*, the fraction of acetate carbon is 0.35, resulting in 2.70 mol of ATP per mol of glucose fermented. For *B. azotofixans*, a similar calculation showed the efficiency factor to be 2.88. Because the data in Table 1 were obtained from one culture for each species, we cannot determine now whether the observed small differences in efficiency factors among the three *Bacillus* species are significant. However, the data show that for all three *Bacillus* species, the efficiency factors fall in the upper range of the theoretical limits of 2 to 3 expected for organisms that ferment glucose by the Embden-Meyerhof pathway. With *C. butyricum*, from 2 mol of acetyl-CoA, the fraction converted to acetate (0.25) yields 2 × 0.25 mol of ATP, but the fraction converted to butyrate (0.75) yields only 0.75 mol of ATP. Butyrate is formed through condensation of 2 mol of acetyl-CoA to 1 mol of acetoacetyl-CoA, reduction to butyryl-CoA, and conversion to butyrate via phosphotransbutyrylase and butyrate kinase, resulting in the regeneration of 1 mol of ATP per 2 mol of acetyl-CoA. Therefore, the efficiency factor for N_2-fixing *C. butyricum* is 2 + (2 × 0.25) + 0.75 = 3.25 mol of ATP per mol of glucose fermented. This value was surprisingly close to the value of 3.3 observed for an ammonia-grown chemostat culture of *C. butyricum* by Crabbendam et al. (5) in which the fractions converted to acetate and butyrate were 0.28 and 0.72, respectively.

ATP/ADP ratios in anaerobic ammonia-grown and N_2-fixing *B. polymyxa* and *B. azotofixans*. Figure 2 shows the separation of adenylates in cell extracts of N_2-fixing cultures of *B. polymyxa* and *B. azotofixans* by ion-pair reversed-phase high-performance liquid chromatography. ADP and ATP peaks were identified by comparing their retention times with those of the respective standard of the nucleotides and confirmed by the observed quantitative increase in the intensity of the assigned peaks when 0.2 nmol each of the standard ADP and ATP was coeluted with the cell extract (Fig. 2B). The AMP peak was similarly identified but was less well resolved. A chromatogram of the culture medium of N_2-fixing *B. polymyxa* from which the cells had been removed by centrifugation showed no peaks for AMP, ADP, ATP, or NAD^+. Therefore, the peaks from these substances (Fig. 2A) arise from intracellular adenylates. Table 2 summarizes the quantities of intracellular ADP and ATP (in nanomoles per milliliter of culture) and the ATP/ADP ratios in N_2-fixing and anaerobic ammonia-grown cultures of *B. polymyxa* and *B. azotofixans*.

To determine whether the ATP/ADP ratios vary during exponential growth in batch cultures, a second N_2-fixing *B. polymyxa* culture was sampled at different times during

TABLE 1. Acetate kinase activities, fermentation products, and the efficiency factors in N_2-fixing cultures

Species	C source	Acetate kinase activity[a]	Fermentation products (mM)[b]						mol of ATP/ mol of glucose
			Acetate	Butyrate	Ethanol	Butanediol	Succinate	Lactate	
B. polymyxa	Sucrose	76	25 (0.35)		33 (0.46)	4.5 (0.12)	2.4 (0.07)		2.70
B. macerans	Sucrose	61	18 (0.32)		38 (0.68)				2.64
B. azotofixans	Sucrose	1,300	57 (0.44)		69 (0.53)			3.1 (0.03)	2.88
C. butyricum	Glucose	262	27 (0.25)	41 (0.75)					3.25

[a] Nanomoles of acetyl phosphate formed per minute per milligram of protein.

[b] The numbers in parentheses represent the fraction defined as (moles of carbon in a specific fermentation product)/(moles of carbon in total non-gaseous fermentation products). For acetate, for example, the fraction represents $Q_A/(Q_A + Q_B + ... + Q_N)$ in equation 3 in Materials and Methods.

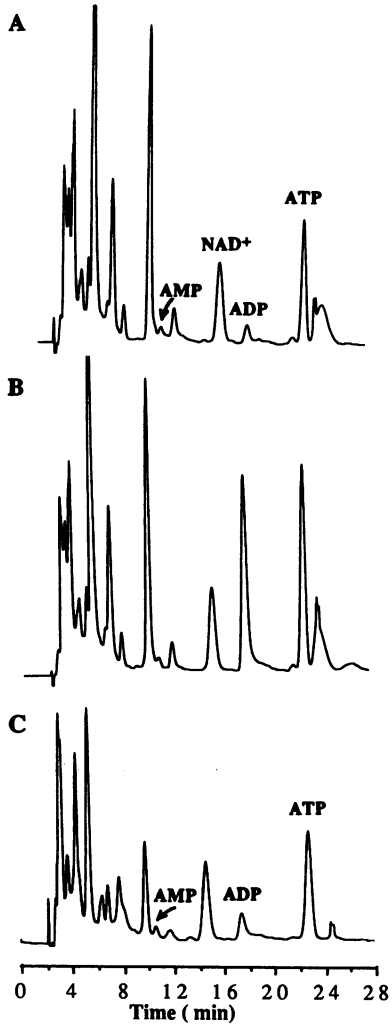

FIG. 2. Separation by ion-pair reversed-phase high-performance liquid chromatography of adenylates in cell extracts of N_2-fixing cultures of *B. polymyxa* (A), *B. polymyxa* coeluted with 0.2 nmol each of standard ATP and ADP (B), and *B. azotofixans* (C).

exponential growth for analysis of the adenylates. The ATP/ADP ratios were 4.6 ± 0.3 and 4.8 ± 0.1 in cultures sampled at 178 and 214 Klett units, respectively. Comparison with the ratio at 230 Klett units (Table 2) shows that the ATP/ADP ratio remains stable during exponential growth and is reproducible in duplicate cultures.

To check whether the ATP/ADP ratio in the anaerobic culture varies when the sampled portion of the culture is exposed to air for a few seconds before quenching, a comparison was made between N_2-fixing *B. polymyxa* cultures quenched with $HClO_4$ (i) by direct anaerobic transfer and (ii) after a 4-s exposure of the surface of the sample to air by allowing the sample to stand in a 5-ml open beaker before the addition of $HClO_4$. The observed ATP/ADP ratios were 4.5 ± 0.18 and 5.0 ± 0.2, respectively. The results suggest that in anaerobically grown cultures of facultative anaerobes in which cytochrome oxidases are often repressed (11), ATP/ADP concentration ratios change much less rapidly on transfer to aerobic conditions than has been observed when aerobic cultures are subjected to anaerobic conditions (16). All data reported unless otherwise indicated, were obtained from cultures quenched anaerobically as described in Materials and Methods.

To investigate whether ATP and ADP are recovered to the same extent when subjected to the extraction procedures, a mixture of ATP and ADP (7.5 nmol each) was added to a 1-ml sample of the N_2-fixing *B. polymyxa* culture at 230 Klett units immediately after sampling and subjected to the same treatment (extraction with $HClO_4$, removal of acid-insoluble material, neutralization, and removal of the precipitates of $KClO_4$) as the cultures used for adenylate extraction. The recovery of added ATP was calculated from {[(total ATP recovered in nanomoles per milliliter of culture) − (ATP extracted from the cell in nanomoles per milliliter of culture)]/[added ATP (7.5 nmol per ml of culture)]} × 100. The recovery of added ATP and ADP was 71 and 72%, respectively, suggesting that some coprecipitation of ATP and ADP with acid-insoluble material and $KClO_4$ occurred. However, the nearly identical percentages of recovery for ATP and ADP show that the adenylates extracted from the cells are very likely to be recovered to the same extent, and therefore the reported ATP/ADP ratios are reliable.

Data in Table 2 show that in the N_2-fixing cultures, the steady-state ATP/ADP ratios are 4.5 for *B. polymyxa* and 3.8 for *B. azotofixans*. In anaerobic ammonia-grown cultures, the ratios are 6.5 and 3.7, respectively. For the N_2-fixing *B. polymyxa* culture at 230 Klett units per ml, the dry weight of cells was 0.67 ± 0.01 mg/ml of culture. Therefore, the ATP pool, after correction for the recovery rate, is 6.3 μmol/g (dry wt). This is within the range of ATP pools of 3 to 10 μmol/g (dry wt) observed in a variety of bacteria (4).

The AMP peaks in the cell extracts of N_2-fixing *B. polymyxa* (Fig. 2A) and *B. azotofixans* (Fig. 2C) were not sufficiently well resolved to permit quantitation, but reasonable estimates from the chromatograms are [AMP] = 0.3[ADP] to 0.7[ADP] for *B. polymyxa* and [AMP] = 0.3[ADP] to 0.5[ADP] for *B. azotofixans*. These ranges are in good agreement with observed AMP/ADP ratios in other bacteria, including nitrogen fixers grown in non-carbon-limited media (26). EC values were calculated on the basis of

TABLE 2. ATP/ADP ratios in anaerobic ammonia-grown and N_2-fixing cultures of *B. polymyxa* and *B. azotofixans*

Species	Nitrogen source	Doubling time (h)	Cells sampled at (Klett units):	Adenylate concn (nmol/ml of culture)[a]		ATP/ADP
				ADP	ATP	
B. polymyxa	NH_4^+	7.6	228	0.49 ± 0.01	3.2 ± 0.09	6.5 ± 0.3
	N_2	25	230	0.67 ± 0.02	3.0 ± 0.11	4.5 ± 0.3
B. azotofixans	NH_4^+	9.0	227	0.97 ± 0.09	3.6 ± 0.18	3.7 ± 0.5
	N_2	9.8	174	0.93 ± 0.03	3.5 ± 0.05	3.8 ± 0.2

[a] For each ammonia-grown culture, samples were analyzed in duplicate, and for each N_2-fixing culture, samples were analyzed in triplicate. The mean values of the concentrations are shown, with the uncertainty expressed in standard deviations of the means, $\sigma_{\bar{x}} = \sigma_x/\sqrt{n}$, where σ_x is the standard deviation and n is the number of determinations.

the observed quantities of ATP and ADP in Table 2 and these estimated quantities of AMP. For N_2-fixing *B. polymyxa*, the estimated EC ranges from 0.86 (if [AMP] = 0.3[ADP]) to 0.81 (if [AMP] = 0.7[ADP]). For N_2-fixing *B. azotofixans*, EC = 0.81 to 0.83 for [AMP] = 0.3[ADP] to 0.5[ADP]. Clearly, EC is not very sensitive to variation in [AMP] when [ATP] » [ADP] > [AMP]. The estimated EC values for N_2-fixing *B. polymyxa* and *B. azotofixans* fall in the range of 0.8 to 0.86.

DISCUSSION

N_2-fixing cultures of the three *Bacillus* species are capable of producing ATP by the acetate-kinase pathway and have efficiency factors of 2.64 to 2.88, which are in the upper range of the theoretical limits of 2 to 3 and comparable to that observed in N_2-fixing *K. pneumoniae* (8). However, these efficiency factors are lower than that of 3.25 observed in N_2-fixing *C. butyricum*. The difference arises from the fact that in the *Bacillus* species, only one of the two major fermentation pathways (the acetate-kinase pathway but not the ethanol pathway) results in additional ATP formation, whereas in *C. butyricum*, both acetate and butyrate pathways contribute to ATP formation. An N_2-fixing culture of *C. pasteurianum*, which has the same fermentation pathways as *C. butyricum* (25), is expected to have an efficiency factor similar to that of *C. butyricum*.

For *K. pneumoniae* which, like *B. polymyxa*, ferments glucose to ethanol, acetate, butanediol, and succinate, the maximal efficiency factor of 3 has been observed only in glucose-limited chemostat cultures (24). When the supply of glucose is limited, maximum efficiency (moles of ATP generated per mole of glucose fermented) ensures a maximum rate of ATP regeneration. In glucose-rich culture, a lower efficiency can be compensated for by a high rate of glucose utilization which permits a rate of ATP regeneration adequate to meet the energy demands of the cell. In the non-carbon-limited N_2-fixing cultures of *B. polymyxa* and *B. azotofixans*, the observed efficiency factors, 2.70 and 2.88, respectively, were less than the optimal 3.0, but the observed ATP/ADP ratios were 3.8 to 4.5, and the estimated EC values were 0.81 to 0.86. These values are very close to the ATP/ADP ratios of 4 to 6 and the EC values of 0.80 to 0.95 typically observed in non-nitrogen-fixing cells growing under nonlimiting substrate conditions. The results suggest that in the N_2-fixing cultures of *Bacillus* spp. studied here, the rate of ATP regeneration is adequate to meet the energy demand for N_2 fixation. Therefore, the rate of ATP regeneration is not growth rate limiting in these cultures.

It is interesting that ATP/ADP ratios are stabilized at normal levels in N_2-fixing *Bacillus* species, whereas in N_2-fixing *K. pneumoniae* and *C. pasteurianum*, which have comparable or higher efficiency factors, much lower ATP/ADP ratios (2.0 and 1.4, respectively) and EC values (0.7 and 0.6, respectively) have been observed under nonlimiting substrate conditions (26). In these cultures, the rate of ATP regeneration was considered to be growth limiting, and the rate of ATP expenditure for N_2 fixation was regulated through partial inhibition of the nitrogenase activity by intracellular ADP.

It is possible that the differences in the ATP/ADP ratios between N_2-fixing cultures of *Bacillus* and *Clostridium* spp. arise, at least partly, from lower rates of ATP expenditure for N_2 fixation and concomitant ammonia assimilation in the *Bacillus* species. The rate of ATP consumption for N_2 fixation depends on the concentration and in vivo activity of nitrogenase as well as the number of moles of ATP consumed per mole of N_2 fixed (apparent ATP/N_2 molar ratio). This ratio remains to be determined for the *Bacillus* species. The observed slow growth of N_2-fixing cultures of *B. polymyxa* (doubling time, 25 h) and *B. azotofixans* (9.8 h) under nonlimiting substrate conditions compared with that of corresponding cultures of *K. pneumoniae* (2.5 h) and *C. pasteurianum* (2.8 h) (25) suggests that the *Bacillus* species, particularly *B. polymyxa*, have low nitrogenase activities. The nitrogenase activity of *B. polymyxa*, as measured by acetylene reduction in an intact culture grown on semisolid medium, is approximately one-sixth of that of *B. azotofixans* (21). We must await measurements of (i) the nitrogenase activities of the *Bacillus*, *Clostridium*, and *Klebsiella* species under the same culture conditions and (ii) the ATP/N_2 molar ratios in the *Bacillus* species in order to evaluate whether the maximum rates of ATP consumption during N_2 fixation are significantly different among these bacteria. In *B. polymyxa*, ammonia produced by N_2 fixation is assimilated into glutamic acid predominantly by the glutamate dehydrogenase pathway without consuming ATP (12). By contrast, in *B. azotofixans* (14), *K. pneumoniae* (19), and *C. pasteurianum* (3, 18, 19), ammonia is assimilated by the ATP-requiring glutamine synthetase-glutamate synthase pathway. If the maximum rate of ATP utilization in the first two reactions of nitrogen assimilation is low in *B. polymyxa* compared with that in *C. pasteurianum* or *K. pneumoniae*, this may account partly for the observed differences in the ATP/ADP ratios among these species.

It is interesting that in anaerobic ammonia-grown *B. polymyxa*, which assimilates ammonia into glutamic acid without expenditure of energy (12), the steady-state ATP/ADP ratio of 6.5 is substantially higher than the ratio of 3.7 observed in the corresponding culture of *B. azotofixans* in which ammonia is mainly assimilated by the ATP-requiring glutamine synthetase-glutamate synthase pathway (14). The steady-state ATP/ADP ratio in each culture reflects the optimum balance between the rates of ATP-regenerating and ATP-utilizing sequences which, in turn, are subject to regulation by adenylates. For a meaningful discussion of the observed difference in ATP/ADP ratios in ammonia-grown cultures, we must await a better understanding of the effects of the ATP/ADP (or AMP) ratio on the activities of regulatory enzymes, such as phosphofructokinase, in these *Bacillus* species.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant DMB85-01617.

We thank Daniel E. Atkinson for a careful reading of the manuscript and helpful suggestions.

LITERATURE CITED

1. **Arbige, M., and W. R. Chesbro.** 1982. Very slow growth of *Bacillus polymyxa*; stringent response and maintenance energy. Arch. Microbiol. **132**:338–344.
2. **Atkinson, D. E.** 1977. Cellular energy metabolism and its regulation. Academic Press, Inc., New York.
3. **Brown, C. M.** 1976. Nitrogen metabolism in bacteria and fungi, p. 170–183. *In* A. C. R. Dean, D. C. Ellwood, C. G. T. Evans, and J. Melling (ed.), Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, England.
4. **Chapman, A. G., and D. E. Atkinson.** 1977. Adenine nucleotide concentrations and turnover rates. Their correlation with biological activity in bacteria and yeast. Adv. Microb. Physiol. **15**:253–306.
5. **Crabbendam, P. M., O. M. Neijssel, and D. W. Tempest.** 1985.

Metabolic and energetic aspects of the growth of *Clostridium butyricum* on glucose in chemostat culture. Arch. Microbiol. **142**:375–382.

6. **Daesch, G., and L. E. Mortenson.** 1967. Sucrose catabolism in *Clostridium pasteurianum* and its relation to N_2 fixation. J. Bacteriol. **96**:346–351.
7. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 209–344. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press, Inc., New York.
8. **Hill, S.** 1976. The apparent ATP requirement for nitrogen fixation in growing *Klebsiella pneumoniae*. J. Gen. Microbiol. **95**:297–312.
9. **Höhn-Bentz, H., and F. Radler.** 1978. Bacterial 2,3-butanediol dehydrogenases. Arch. Microbiol. **116**:197–203.
10. **Ingebretsen, O. C., A. M. Bakken, L. Segadal, and M. Farstad.** 1982. Determination of adenine nucleotides and inosine in human myocard by ion-pair reversed-phase high-performance liquid chromatography. J. Chromatogr. **242**:119–126.
11. **Jurtshuk, P., T. J. Mueller, and W. C. Acord.** 1974. Bacterial terminal oxidases. Crit. Rev. Microbiol. **3**:399–468.
12. **Kanamori, K., R. L. Weiss, and J. D. Roberts.** 1987. Ammonia assimilation in *Bacillus polymyxa*: N-15 NMR and enzymatic studies. J. Biol. Chem. **262**:11038–11045.
13. **Kanamori, K., R. L. Weiss, and J. D. Roberts.** 1987. Role of glutamate dehydrogenase in ammonia assimilation in nitrogen-fixing *Bacillus macerans*. J. Bacteriol. **169**:4692–4695.
14. **Kanamori, K., R. L. Weiss, and J. D. Roberts.** 1988. Glutamate biosynthesis in *Bacillus azotofixans*. J. Biol. Chem. **263**:2817–2823.
15. **Kanamori, K., R. L. Weiss, and J. D. Roberts.** 1989. Ammonia assimilation pathways in nitrogen-fixing *Clostridium kluyverii* and *Clostridium butyricum*. J. Bacteriol. **171**:2148–2154.
16. **Klofat, W., G. Picciolo, E. W. Chappelle, and E. Freese.** 1969. Production of adenosine triphosphate in normal cells and sporulation mutant of *Bacillus subtilis*. J. Biol. Chem. **244**:3270–3276.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Mortenson, L. E.** 1978. Regulation of nitrogen fixation. Curr. Top. Cell. Regul. **13**:179–232.
19. **Nagatani, H., M. Shimizu, and R. C. Valentine.** 1971. The mechanism of ammonia assimilation in nitrogen fixing bacteria. Arch. Mikrobiol. **79**:164–171.
20. **Nakajima, H., K. Suzuki, and K. Imahori.** 1978. Purification and properties of acetate kinase from *Bacillus stearothermophilus*. J. Biochem. **84**:193–203.
21. **Seldin, L., J. D. Van Elsas, and E. G. C. Penido.** 1984. *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. Int. J. Syst. Bacteriol. **34**:451–456.
22. **Stouthamer, A. H., and C. Bettenhaussen.** 1972. Influence of hydrogen acceptors on growth and energy production of *Proteus mirabilis*. Antonie van Leeuwenhoek **38**:81–90.
23. **Streekstra, H., E. T. Buurman, C. W. G. Hoitink, M. J. Teixeira de Mattos, O. M. Neijssel, and D. W. Tempest.** 1987. Fermentation shifts and metabolic reactivity during anaerobic carbon-limited growth of *Klebsiella aerogenes* NCTC 418 on fructose, gluconate, mannitol and pyruvate. Arch. Microbiol. **148**:137–143.
24. **Teixeira de Mattos, M. J., and D. W. Tempest.** 1983. Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat culture. Arch. Microbiol. **134**:80–85.
25. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100–180.
26. **Upchurch, R. G., and L. E. Mortenson.** 1980. In vivo energetics and control of nitrogen fixation: changes in the adenylate energy charge and adenosine 5′-diphosphate/adenosine 5′-triphosphate ratios of cells during growth on dinitrogen versus growth on ammonia. J. Bacteriol. **143**:274–284.
27. **Walker-Simmons, M., and D. E. Atkinson.** 1977. Functional capacities and the adenylate energy charge in *Escherichia coli* under conditions of nutritional stress. J. Bacteriol. **130**:676–683.
28. **Weimer, P. J.** 1984. Control of product formation during glucose fermentation by *Bacillus macerans*. J. Gen. Microbiol. **130**: 103–111.
29. **Wood, W. A.** 1961. Fermentation of carbohydrates and related compounds, p. 59–150. *In* I. E. Gunsalus and R. Y. Stanier (ed.), The bacteria. II. Metabolism. Academic Press, Inc., New York.